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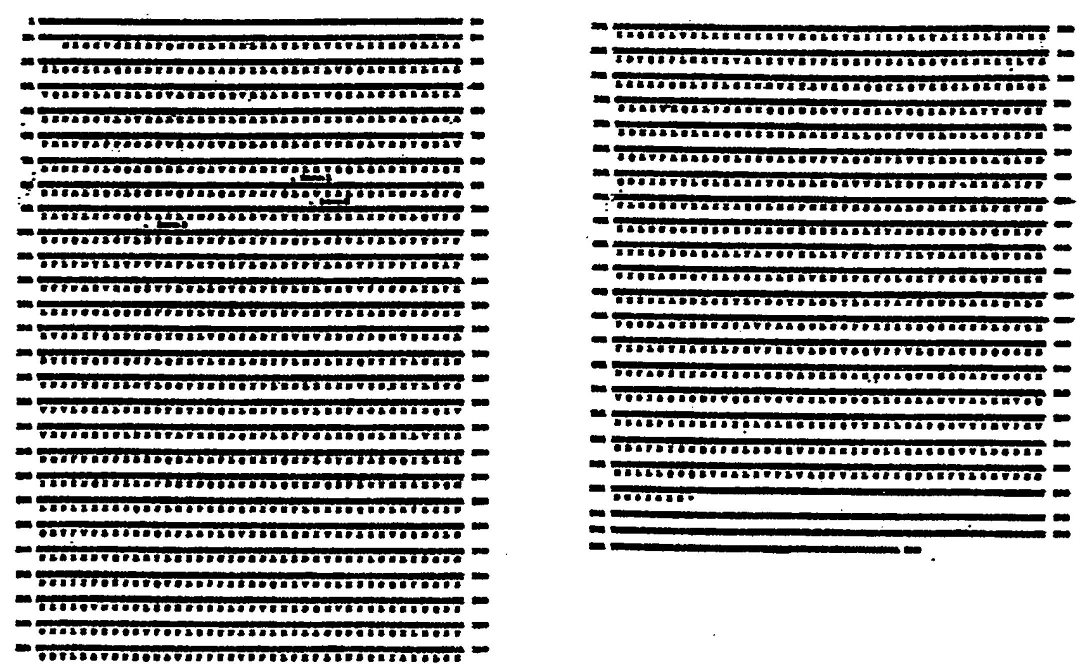

A gene encoding a protein, nesting, whose expression distinguishes neural multipotential stem cells and brain tumor cells from the more differentiated neural cell types (e.g., neuronal, glial and muscle cells). Methods of detecting the expression of nestin as a means of diagnosing and treating brain tumours are also described.

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# NESTIN EXPRESSION AS AN INDICATOR OF NEUROEPITHELIAL TUMORS

## Description

### Background

Brain tumors are a leading cause of cancer deaths in people under the age of 35. An important concept in our understanding of brain tumors is that the neoplastic cells may arise from undifferentiated neuroectodermal cells (Rubinstein, L.J. J. Neurosurg 62: 795-805 (1985)).

At the gastrula stage of central nervous system (CNS) development, neuroectoderm, forming the neural plate is derived from ectoderm which has been induced by underlying mesoderm. (Spemann, H. Yale University Press 1938)). The neural plate then folds, in the process called neurulation, to form the neural tube. The cells of the neural tube are the precursors for the major differentiated cell types in the central nervous system: astrocytes, oligodendrocytes, and the various types of neuron (Sauer, F.C. J. Comp. Neurol. 62: 377-405 (1935)). The cells of the neural tube may also be the precursors to neoplastic cells which form brain tumors.

Recent success in "tagging" individual CNS precursors and their daughter cells, either through infection by a marker-bearing retrovirus or by injection of stable, specific dyes and enzymes, has shown that neurons and non-neuronal cells (i.e., glia cells) are often derived from a common precursor. In retina a common precursor for neurons and the Mueller glial cell exists

very late in development (Holt, C.E., T.W. Bertsch et al., Neuron 1: 15-26 (1988); Turner, D.L. and C.L. Cepko, Nature 238: 131-136 (1987); Wetts, R. and S.E. Fraser, Science 239: 1142-1145 (1988); a common precursor for different neuronal and glial cell types has also been demonstrated in cortex and tectum (Luskin, M.B., A.L. Pearlman et al., Neuron 1: 635-647 (1988); Price, J. and L. Thurlow, Development 104: 473-482 (1988); Walsh, C. and C. Cepko, Science 241: 1342-1345 (1988)).

Tissue culture systems have provided additional information about the differentiation potential of CNS stem cells. Single-cell microculture of embryonic day 14 rat forebrain has produced clones containing both neurons and glia (Temple, S. Nature 340: 471-473 (1989)). Analysis of a later precursor in the glial differentiation pathway has established specific factors influencing differentiation to oligodendrocytes or type II astrocytes (Raff, M Science 243: 1450-1455 1989)). Cell lines established by immortalization of CNS stem cells also reflect features of the stem cell and can differentiate along both the neuronal and glial pathways (Cepko, C.L. Ann. Rev. Neurosci. 12: 47-65 (1989); Frederiksen, K. et al., supra (1988)).

Antibodies have also proven useful for analyzing stem cells. For example, antibodies A2B5 (Raff, M.C. supra 1989) D1.1 (Levine, J.M., L. Beasley et al., J. Neurosci 4: 820-831 (1984), Rat 401 (Hockfield, S. and R. McKay, J. Neurosci 5: 3310-3328 (1985), and antisera against the intermediate filament vimentin (Bignami, A., T. Raju et al Dev. Biol 91: 286-295 (1982); Federoff, S.

Molecular Bases of Neural Development (1985); Tapscott, S.J., G.S. Bennett et al., Dev. Biol. 86: 40-54 (1981) bind to antigens enriched in the proliferative zone of the neural tube. However, A2B5 and D1.1 recognize glycolipid epitopes and are less useful for analysis of gene expression. Vimentin, which appears transiently in brain development, is an ambiguous marker largely because of its promiscuous expression in many cultured cells as well as in a variety of developing and differentiated tissues (Traub, N. Intermediate filaments: a review. Berlin, SpringerVerlag (1985)).

The correlation between Rat 401 and proliferating cells in the developing nervous system has been examined in detail. Rat 401 was found to recognize a transient population of embryonic columnar epithelial cells and radial glial cells in many regions of the CNS (Hockfield, S. and McKay J. Neurosci. 5: 3310-3328 (1985)). The number of Rat 401 positive cells, their proliferative rate, and the developmental kinetics of Rat 401 expression relative to neuronal differentiation revealed that the immediate precursors to neurons are also Rat 401 positive (Frederiksen and McKay,J. J. Neurosci 8: 1144-1151 (1988)). Neither neurons nor glia in the adult brain express the epitope recognized by the Rat 401 antibody.

Although "tagging", tissue culture systems and antibodies have proven useful in analyzing neural precursor cells, there is still an incomplete understanding of the lineage of tumor development. For example, medulloblastoma, a common brain tumor of children, is associated predominantly with the cerebellum and brain

stem. Medulloblastoma tumors contain cell types with differentiated characteristics of neurons, glia and muscle (Rubinstein, supra 1985; Coakham H.B., et al., J. Clin. Pathol. 38: 165-173 (1985); Velasco, M.E. et al., Surg. Neurol. 23: 177-182 (1985); Hayashi, K. et al., Acta Pathologica Japonica 37: 85-96 (1987); Cras, P. et al., Acta. Neuropathol 75: 377-384 (1988)).

Because of the presence of multiple differentiated cell types found in these tumors, Bailey and Cushing (Bailey, P. and H. Cushing, Arch. Neurol. Psychiatry 14: 192-224 (1925) proposed that rather than being multiple coincident tumors, they were derived from a multi-potential stem cell, which they called a "medulloblast". According to this model, a medulloblastoma tumor would result from uncontrolled proliferation and differentiation of the medulloblast. However, the hypothetical medulloblast has not been identified.

Due to the incomplete understanding of brain tumor development, there is currently no adequate assay for detecting brain tumors at an early stage in their development. In addition, there is no specific noninvasive method of treating brain tumors. Methods of detecting and treating brain tumors would be very useful.

## Summary of the Invention

The present invention relates to a gene encoding a protein whose expression distinguishes neural multi-potential stem cells and brain tumor cells from the more differentiated neural cell types (e.g. neuronal, glial and muscle cells) of the mammalian brain.

The present invention relates to the nestin gene, particularly a nestin gene of mammalian origin, and the encoded nestin protein. As described herein, two nestin genes of mammalian origin, the rat nestin gene and the human nestin gene, have been isolated and sequenced. The coding sequence of the rat nestin gene transcript is 5415 bp, which corresponds to an expected molecular weight of approximately 200 kD for the nestin protein. The relative molecular weight of the nestin protein as determined by SDS polyacrylamide gels is approximately 240 kD. The predicted amino acid sequence of the rat nestin gene product shows that nestin defines a distinct sixth class of intermediate filament protein.

The present invention also relates to the human nestin gene and the encoded human nestin protein. The coding sequence of the human nestin gene transcript is 4854 bp. The predicted amino acid sequence is 1618 amino acids in length, and shows significant sequence homology with the rat nestin sequence.

The present invention further relates to methods of detecting the expression of nestin as a means of diagnosing a predisposition to the development of a brain tumor or the presence of a brain tumor in an adult individual. In one embodiment, DNA present in a sample from the brain of an adult individual is hybridized to a DNA probe which is complementary to all or a portion of the nestin gene. As used herein, the term the nestin gene includes the human nestin gene whose sequence is represented herein, the rat nestin gene whose sequence is also represented herein and equivalent genes from other

species, such as those which are substantially homologous to the human or the rat gene sequence. Detection of hybridization is an indication of a predisposition to the development of a brain tumor or the presence of a brain tumor. In another embodiment, cerebral spinal fluid or a serum sample from the brain of an adult individual can be stained with anti-nestin antibodies. Detection of stained cells is an indication of a predisposition to the development of a brain tumor or the presence of a brain tumor.

## Brief Description of the Drawings

Figure 1 is the nucleotide and deduced amino acid sequences of the nestin transcript derived from the central nervous system of rat embryos. The three intron positions (912, 1038 and 1111) are indicated. The canonical poly A addition signal (5924-5929) is underlined.

Figure 2 is the nucleotide sequence of human nestin.

Figure 3 is the deduced amino acid sequence of human nestin.

## Detailed Description of the Invention

The present invention relates to a gene of mammalian origin encoding a protein whose expression distinguishes neural multipotential stem cells and brain tumor cells from the more differentiated neural cell types (e.g., neuronal, glial and muscle cells of the adult brain).

The present invention more particularly relates to the nestin gene and the encoded proteins and their use in

diagnosing tumors of the brain, such as medulloblastomas, gliablastomas and oligodendroglioma. As described herein, the human nestin gene and the rat nestin gene have been isolated and sequenced. In addition, expression of the nestin gene and its structural features are described. The intracellular distribution of the nestin gene product, both in an embryonic rat brain cell line (ST15A) and human brain tissue, including tumor cells, using immunocytochemical and immunohistochemical techniques is also described. Finally, nestin expression in the developing human central nervous system and brain tumor samples is also described herein.

## CLONING AND CHARACTERIZATION OF THE RAT AND HUMAN NESTIN GENE

### The Rat Nestin Gene

A cDNA library in the expression vector lambda gt11 was constructed from poly(A)$^+$ RNA from CNS of embryonic day 15 (E15) rat embryos as described in detail in Example 1. The library was screened with the monoclonal antibody Rat 401 after induction of protein expression and immunopositive plaques were identified. The cDNA insert from the most immunopositive clone, gt11.401.19, was used as probe to isolate additional clones, gt10.401:16, gt10.401:18 and gt10.401:9, in a lambda gt10 library from rat E15 CNS constructed in parallel with the gt11 library. A number of hybridizing clones were identified and sequenced and together represent 5333 base pair (bp) of transcribed sequence, including 404 bp of 3'

untranslated sequence and a consensus polyA addition site.

The remaining portion of the nestin gene was obtained from genomic sequences. Genomic Southern blots, even when hybridized at low stringency, revealed a pattern consistent with a single rat gene. A genomic rat library in the vector Charon 4A was screened with the nestin cDNA clones. Hybridizing clones were organized by restriction mapping and the region upstream of the cDNA clone gt10.401:9 was sequenced.

An open reading frame continues for an additional 600 bp from the 5′ end of the most upstream cDNA clone. This region from the genomic clone Cha.401:14 was subdivided into three different fragments with the restriction enzyme BamH1. These three fragments all hybridized to an mRNA of the correct size, 6.2 kb, when used as probes on Northern blots with rat E15 CNS RNA.

The exact location of the 5′ end was determined by a combination of S1 nuclease mapping and primer extension. A probe (bp -121 to +194 relative to +1 - the subsequently defined transcription start) was generated overlapping the end of the genomic open reading frame by polymerase chain reaction between two specific primers. This PCR product protected a 194 bp fragment in S1 nuclease analysis. Primer extension from the downstream primer used to generate the PCR fragment also produced a 194 bp fragment. The fact that these two experiments gave the same fragment suggests that the transcription start site has been defined.

Figure 1 shows the nucleotide sequence of the rat nestin gene. The total length of the nestin gene transcript is 5945 bp, excluding the poly A tail.

There are two potential initiation codons at positions +127 and +160. Assuming the first methionine is the initiation codon, the coding sequence is 5415 bp. This corresponds to an expected molecular weight of approximately 200 kD; the relative molecular weight as determined by migration on SDS polyacrylamide gels is approximately 240 kD (Hockfield and McKay, supra (1985)). Sequence comparison of cDNA and genomic clones reveals three introns at positions 912, 1038, and 1111 in the transcribed region.

To confirm that the cloned gene indeed represents the gene encoding the Rat401 antigen, a synthetic peptide from the C-terminal 20 amino acids was made. The extreme C-terminus was chosen because it is not included in the clone recognized by the original monoclonal antibody, and therefore represents a distinct epitope. A polyclonal antiserum against the peptide was raised in rabbits and identifies a band in Western blots and a pattern in immunostaining experiments which are indistinguishable from that recognized by the monoclonal antibody Rat 401.

Structural Features of the Rat Nestin Gene

The sequence of the entire transcribed region of the rat nestin gene was compared to the Genbank and EMBL databases. The only significant similarities found are to the five classes of intermediate filaments: acidic keratins (class I); basic keratins (class II); desmin, glial fibrillary acidic protein, peripherin, and vimentin (class III); the neurofilament triplet (class IV); and nuclear lamins (class V) (Steinert and Roop, 1988)).

The similarity between the nestin gene and the genes of the other five classes of intermediate filaments ranges from 16 to 29 % at the amino acid level in a 307 amino acid long region starting close to the N-terminus of the nestin gene, corresponding to the conserved alpha-helical rod or "core" domain of the intermediate filaments. This region of the predicted nestin amino acid sequence also contains a repeated hydrophobic heptad motif characteristic of intermediate filaments. The degree of amino acid similarity is comparable to that found between different classes of intermediate filament, both in degree and in location, i.e., conserved heptad-containing alpha-helical stretches within the core domain are separated by less well conserved, non-heptad spacers (Steinert, P.M.and D.R. Roop, Ann. Rev. Biochem. 57: 593-625 (1988)). The locations of the nestin gene's three introns are not conserved with respect to those of other classes of intermediate filaments.

In the regions outside the conserved rod domain, no strong similarities to other characterized genes were found. The N-terminal domain is only 11 amino acid residues long, shorter than in other intermediate filaments. The large C-terminal domain loosely resembles those of neurofilaments in that it is highly charged, bears glutamate-rich regions, and features a repeat, in this case the 11 amino acid motif S/P-L-E-E/K-E-X-Q-E-S/L-L-R (underlined residues are strongly conserved). There are approximately 35 of these repeats in the region between amino acids 512 and 1050.

The deduced amino acid sequence of nestin suggests that it is a member of the intermediate filament protein

family. However, its degree of sequence homology to other intermediate filaments in the core domain, its dissimilarity in the head and tail domains, and its different splicing pattern suggest that it defines a new class of intermediate filament protein.

The Human Nestin Gene

As described in detail in Example 5, the human nestin gene was isolated using low stringency DNA hybridization of a human genomic bacteriophage lambda library with a rat nestin probe.

Figure 2 shows the nucleotide sequence of the human nestin gene. The total length of the human nestin gene sequence is 4854 bp. Figure 3 shows the deduced amino acid sequence encoded by the human nestin gene which is 1618 amino acid residues in length. The alignment of the deduced amino acid sequences of human nestin with the deduced amino acid sequence of rat nestin showed greater than 75% sequence similarity between the two sequences, with greater than 60% sequence identity.

DEVELOPMENTAL EXPRESSION OF THE RAT AND HUMAN NESTIN GENE AND ENCODED PRODUCT

Developmental Expression of the Rat Nestin Gene

During development, CNS stem cells differentiate into neurons and glia on a stereotyped schedule; different brain regions become post-mitotic at different times. The nestin protein was originally identified by the Rat 401 antibody, which transiently stains radial

glial cells of the neural tube in cross sections of rat embryos (Fredericksen, K. and R. McKay, J. Neurosci 5: 3310-3328 (1988); Hockfield and McKay, supra (1985)). The distribution of staining in the neural tube at different developmental stages, combined with quantitative double-label experiments using tritiated thymidine autoradiography and FACS cell cycle analysis, showed that expression of the Rat 401 antigen was localized to proliferating CNS stem cells during embryogenesis (Frederiksen and McKay, supra (1988)) and U.S. Patent Applications Serial Nos. 180,548; 201,762 and 603,803 herein incorporated by reference. These experiments have been extended by analyzing the developmental expression of the nestin gene.

Northern blots of RNA from E15 rat CNS or from the Rat 401 positive cell line ST15A were probed with nestin cDNA inserts, a single 6.2 kb mRNA species was identified. To investigate the temporal profile of nestin RNA expression in the developing CNS, representatives of early (cerebrum) and late (cerebellum) developing regions of the brain were compared.

In cerebrum, nestin expression was found to decline from a maximum on day E16, such that only extremely low levels of RNA could be detected at day P9 and P12 and none at all in the adult cerebral cortex, even after long exposures. The loss of nestin mRNA precisely parallels the decline of Rat 401 positive stem cells in cerebrum during neurogenesis (Frederiksen and McKay, supra (1988)). The postnatal cerebellum expresses the nestin gene with a maximum on day P5; no expression can be

detected after day P9 . Again, this pattern closely matches the numbers of Rat 401 positive cells in developing cerebellum.

Nestin is not detected by Northern blot analysis in adult tissue. The loss of nestin expression coincides with terminal differentiation of these early multi-potential cells. There are two important implications of these results. First, nestin appears to be a general marker for the CNS stem cell from the newly closed E11 neural tube through the postnatally developing cerebellum. Second, nestin expression is a consequence of transcriptional regulation of the gene.

The only expression of nestin observed outside developing CNS is traces of nestin message from day P4 developing skeletal muscle. This finding is consistent with previous observations from cross sections of rat embryos, where Rat 401 staining in the developing somites was observed (Hockfield and McKay, supra (1985)). Developing nervous tissue and developing skeletal muscle may share unexpected kinship with regard to their potential fates.

<u>Intracellular Distribution of the Nestin Gene Product in the ST15A Cell Line</u>

The associations of nestin with different components of the cytoskeleton were analyzed by a series of double-label immunocytochemistry experiments. These experiments were carried out in the immortalized CNS precursor cell line ST15A which expresses, in addition to components of microtubules and microfilaments, nestin and the inter-

mediate filament vimentin. ST15A produces a nestin mRNA indistinguishable by Northern blot analysis from that found in E15 CNS.

In the first set of double-label experiments the intracellular distribution of the nestin gene product, detected by the monoclonal Rat.401 antibody and an appropriate second antibody, was compared to the distribution of actin, tubulin and vimentin. The latter two proteins were detected by polyclonal antibodies with matching second antibodies while the actin pattern in microfilaments was visualized by rhodamine-conjugated phalloidin.

The intracellular distributions of nestin, microtubules (tubulin), and microfilaments (actin) are quite different. Microtubules are more evenly distributed in the cell with no particular aggregation around the nucleus, whereas nestin is found in a fiber-like pattern, with a pronounced perinuclear accumulation. We also observe distinct differences when microfilaments and nestin are stained in the same cell: microfilaments form straight and parallel fibers, in contrast to the gently curved fibers of nestin.

Differences in nestin and vimentin staining in the same ST15A cells are much less clear. Vimentin staining is stronger than nestin in the cell periphery, and there are regions where intensity varies between the two proteins, but common features are also evident. The overall organization of the two networks is quite similar, with filament arrays radiating out from a perinuclear center. Moreover, there are regions in cells where it seems likely that nestin and vimentin colocalize.

In another set of experiments, microfilaments and microtubules were disrupted by cytochalasin B and colchicine, respectively, and the effects on nestin distribution analyzed. When ST15A cells were treated with 10 ug/ml cytochalasin B for one hour the microfilaments became highly disorganized, while the overall morphology of the nestin network was preserved. Microtubules also remained intact after the cytochalasin B treatment. Colchicine treatment (25 ug/ml) for 24 hours caused a collapse of the microtubules. The nestin network could still be identified, although the perinuclear distribution became much more prominent. A similar pattern was observed for vimentin as has been previously reported (Hynes, R.O. and A.T.Destree, Cell 13:151-163 (1978): Monteiro, M.J. and D.W. Cleveland, J. Cell Biol. 108: 579-593 (1989). Microfilaments were essentially unaffected by the colchicine treatment. The inferred relationship of nestin to intermediate filaments based on its deduced amino acid sequence is thus supported by the intracellular distribution of the nestin gene product under various conditions.

ST15A Cells have Features Expected of a Neural Precursor to Brain Tumors

"The prevailing name 'medulloblastoma' given by Bailey and Cushing (1925), is unfortunate because there is no embryonal cell that has been identified as a medulloblast." This sentence begins the discussion of medulloblastoma in a prominent text on neural tumors (Russell, D. and L.J.Rubinstein, Pathology of Tumors of

<u>the Nervous System</u>, Baltimore, Williams and Wilkins (1989)). The following presents evidence that a cell line derived from rat cerebellum, ST15A, has features expected of a medulloblast.

ST15A was derived from postnatal day 4 rat cerebellum. In the rat, as in other mammals, much of cerebellar development occurs after birth, so that there are a large number of dividing precursor cells present at the time from which this line was derived. Thus, ST15A is derived from the appropriate tissue and stage of development. It has previously been shown that ST15A cells can differentiate along neuronal and glial pathways. The following experiments, which are described in detail in Example 3, show that ST15A cells can also differentiate into muscle. This result suggests that a single neuroectodermal cell can give rise to the different cell types found in brain tumors.

The cerebellar cell line ST15A was one of several cell lines obtained by infecting primary rat cerebellar cells with a recombinant retrovirus carrying SV40 T-antigen. A temperature sensitive allele of T-antigen was used to establish these cell lines. At the permissive temperature (i.e., when the T-antigen protein is active) the cells proliferate and express the intermediate filament protein nestin.

At the non-permissive temperature the T-antigen protein is rapidly degraded and the cells differentiate into either neurons or glial cells depending on the conditions. The differentiation into neurons and glial cells is best accomplished by growing ST15A cells in

co-culture with primary cerebellar cells. Partial differentiation has also been observed when ST15A cells are cultured alone; serum-free medium promotes neuronal differentiation and medium containing fetal calf serum promotes glial differentiation.

ST15A cells also can differentiate into muscle cells. This fate was first noted when spontaneously contracting cells were observed in long term cultures of ST15A at 39°C, the non-permissive temperature for T-antigen. Horse serum is often used to support the differentiation of primary myoblasts. When ST15A cells are grown in horse serum at 39°C, they reproducibly differentiate into multi-nucleate cells which express muscle specific proteins, regenerative action potentials and spontaneously contract.

ST15A cells grown for two days at 33°C in 10% fetal calf serum (FCS) in DMEM grew in a disorganized manner; in contrast ST15A cells grown at 33°C in 10% horse serum at 39°C became aligned. The monoclonal antibody Rat 401 recognizes the 220 kD intermediate filament protein nestin. ST15A cells express nestin at both 33°C and 39°C. A second monoclonal antibody which recognizes a skeletal and cardiac muscle isotype of troponin T was reacted with the cells (Lin, J. Biol. Chem. 263: 7309 (1988)). Immunofluorescence shows that troponin T expression is induced when ST15A cells are cultured at 39°C in horse serum.

Immunoblots of proteins extracted from ST15A cells verify that anti-troponin T antibody recognizes a strong band of 38 Kd, the appropriate molecular weight, in the

cells grown at 39°C in horse serum; at 33°C no band is present. The troponin band in ST15A co-migrates with troponin extracted from rat postnatal day 4 muscle. The double band seen in both muscle cells and differentiated ST15A cells may represent the adult and embryonic isoforms of troponin T which differ by 3,000 daltons (Lin, J. Biol. Chem. 263: 7309 (1988)). These two isoforms are derived from the same primary transcript by differential splicing. Lanes carrying the same amount of protein were also probed for nestin with the monoclonal antibody Rat 401. Nestin is present at both temperatures but at elevated levels at the higher temperature. The lower bands are proteolytic fragments of nestin which are often present in protein extracts even in the presence of protease inhibitors. Nestin is also present in postnatal rat muscle.

A family of DNA binding factors has been shown to play a critical role in the differentiation of muscle. These proteins, myoD1, myogenin, myf5 and herculin/MRF4, contain DNA binding motifs of the helix-loop-helix type in a region of homology to c-myc (Wright, W.E., et al., Cell. 56: 607 (1989); Miner, J.H. and B.Wold, Proc. Nat. Acad. Sci. 87: 1089-1093 (1990); Davis, R.L. et al., Cell. 51: 987-1000 (1987); Braun, T et al., EMBO J 8: 701-709 (1989); Rhodes S.J.and S.F.Konieczny, genes and dev. 3: 2050-2061 (1989). The expression of these factors at the mRNA level in ST15A cells as they differentiate into muscle was examined.

Northern blot analysis of total RNA from differentiating ST15A cells probed with the indicated sequences was performed. Each probe hybridized only to the bands

of the expected mobilities. MyoD1 is expressed in passaging ST15A cells at the permissive temperature for the T antigen and continues to be expressed when the cells are shifted to 39°C. Myogenin is not expressed under normal growth conditions but is expressed by 2 days after confluent cells are shifted to 39°C in horse serum (HS); if confluent cells are placed in HS and held at 33°C, the induction of myogenin expression is dramatically inhibited.

Herculin/MRF4 was found in adult skeletal muscle but was not detected in ST15A. Myf5 gave a very weak signal in ST15A cells compared to the myoblast cell line C2C12. Nestin mRNA accumulates with time at 39°C, as does the protein. β-actin serves as a control for the amount of RNA at 33°C and in the short time points at 39°C ; the decrease in β-actin signal at 3 and 7 days at 39°C may be due to the changing composition of the cytoskeleton accompanying differentiation, as levels of myoD1 mRNA appear constant throughout the experiment. These results indicate the ST15A cells express myogenic transcription factors and that the induction of myogenin correlates with the differentiation into the muscle fate.

As expected of muscle cells, ST15A cells become multinucleate as they differentiate. The most striking similarity to muscle, which initiated this series of experiments, was that ST15A cells grown to confluence in FCS at 33°C and cultured for up to 15 days at 39° in 10% horse serum can contract spontaneously. The initial action potentials were generated by a series of hyper-polarizing pulses. The final hyper-polarizing pulse was followed by a train of spontaneous action potentials. Spontaneous action potentials were seen after 12 days at 39°C in 10% horse serum.

Coincident with the appearance of contracting cells, the resting membrane potential falls and it becomes easier to stimulate trains of action potentials with hyperpolarizing current pulses. ST15A cells also twitched in response to stimuli which elicited action potentials. These results show that ST15A cells differentiate into muscle, expressing myogenic transcription factors, muscle-specific cytoskeletal components and electrical excitability.

An interesting question raised by the properties of ST15A cells is whether primary cerebellar cells can differentiate into the muscle fate. Dissociated cells from postnatal day 5 rat cerebellum were cultured for 6 days under conditions which promote muscle differentiation of ST15A cells (39°C in the presence of 10% horse serum) and stained with monoclonal antibody against troponin T. These culture conditions promote extensive differentiation of neurons and glia. Troponin T positive cells with an elongated, multinucleate morphology of muscle cells were also seen, but these cells were very rare, occurring less than one per hundred thousand cells.

Developmental expression of Human Nestin Gene

To characterize human nestin and determine the timing of its induction in the normal human developing CNS, studies were carried out, to examine this class VI IF protein in the normal human developing central nervous system (CNS), human brain tumor derived cell lines, and tissue samples of human CNS tumors.

Human nestin exhibited biochemical and immunochemical properties similar to those of rat nestin.

Further, in the human, nestin was detected immunohistochemically in several different types of immature human CNS cells, i.e. germinal matrix cells, neuroepithelial cells lining the central canal, radial glia and endothelial cells. Nestin appeared in these cells at the earliest gestational age (i.e., 6 weeks) examined here and then it declined in all but the endothelial cells at later embryonic stages. Nestin also was detected by immunocytochemistry in 6 of 7 primitive neuroectodermal tumor cell lines and in both of the malignant glioma cell lines examined. In these cell lines, nestin co-localized incompletely with bundles of IFs containing other IF proteins (i.e. vimentin, glial filament, nuerofilament). Nestin was ubiquitous in a wide variety of brain tumors, but was most prominent in gliomas. These studies document the existence of a human counterpart of rat nestin.

<u>Distribution of the Nestin Gene Product in the Human Developing CNS</u>

Cervical levels of six developing human spinal cords with gestational ages (GAs) of 6 to 40 weeks were immunohistochemically stained with anti-nestin 129. The spinal cord of the 6 weeks GA human fetus was composed of three distinct layers, i.e. a layer of primitive neuroepithelial cells lining the central canal, a mantle layer and an outer marginal layer. Anti-nestin 129 stained most cells in the primitive neuroepithelial layer, i.e. the presumptive miltipotential stem cells that give rise to CNS neurons and glia. In addition, thin elongated radial glial fibers extending from the primitive neuro-

epithelial layer to the subpial region were stained by this antiserum. These neuroepithelial stem cells and radial glial fibers also expressed vimentin but not GFAP. Neuroblasts in the mantle layer (identified by their morphology and NF protein positively) did not express nestin.

At 11 weeks GA, cells in the ependymal lauer were positively stained with anti-nestin 129, as were radial glial fibers. These radial glial fibers were also stained with the anti-vimentin MAb. Nestin immunoreactivity decreased at 17 weeks GA and disappeared at 20 weeks GA. From this gestational age to maturity, GFAP positive glial cells and NF positive neurons were seen in the spinal cord, but no elements other than endothelial cells were nestin positive.

The telencephalic germinal matrix of two cases with GAs of 17 and 20 weeks were examined with anti-nestin 129. At both time points, many presumptive neuroepithelial stem cells in the germinmal matirx cells were nestin positive. These neuroepithelial precursor cells also were labeled by the anti-vimentin MAb, but no germinal matrix cells were stained by the MAbs to GFAP or NF proteins. At 40 weeks GA, a well defined germinal matrix was not evident, but clusters of morphologically immature cells (presumably residual, multipotential precursor cells) were seen in the subependymal region. Nestin immunoreactivity was recognized in only a few of these cells.

To determine if neuroepithelial precursors in the cerebellum also expressed nestin, four developing human cerebelli at GAs of 17 to 40 weeks were studied. Late in

development, the cerebellar cortex has four distinct layers, i.e. the internal and external granular layers, the Purkinje cell layer and the molecular layer. However, these layers were not yet evident at 17 weeks GA and Purkinje cells were not recognizable by morphological criteria. Nevertheless, immature NF positive cells were observed at 17 weeks GA in the superficial internal granular layer, and these cells probably correspond to nascent Purkinje cells. Nestin immunoreactivity was detected at this time in radial glial cells in the internal granular layer and in radial glial processes that extended to the external granular layer. These radial glial cells, which mature into the Bergmann glia of the adult cerebellum, also were stained by the anti-vimentin MAb (data not shown), but not by the anti-GFAP MAb. However, the anti-GFAP MAb did stain ependymal cells lining the fourth ventricle. At 20 weeks GA, nestin immunoreactivity in radial glia was markedly diminished and by 40 weeks GA, it was weakly present in only a few radial glia fibers, while the blood vessel endothelial cells at 40 weeks GA were clearly nestin positive. In contrast, radial glial fibers expressed both GFAP and vimentin (data not shown) at these time points.

Nestin Expression in Brain Tumor-derived Cell Lines

Indirect immunofluorescence studies of 9 well characterized human brain tumor derived cell lines showed variable nestin immunoreactivity in 6 or 7 primitive neuroectodermal tumor (PNET) cell lines, and very intense nestin staining in 2 glioma cell lines. Table 1

summarizes the data on the expression of nestin and other IF proteins in these cell lines.

TABLE 1

IMMUNOFLUORESCENCE ASSAY OF BRAIN TUMOR-DERIVED CELL LINES

| CELL LINES | NESTIN | VIMENTIN | GFAP | NF-L | NF-M | NF-H |
|---|---|---|---|---|---|---|
| Daoy | - | + | - | - | - | - |
| D283 Med | + | + | - | + | + | + |
| D341 Med | + | + | - | - | + | + |
| D384 Med | + | + | - | + | + | + |
| D425 Med | + | + | - | + | + | + |
| D458 Med | + | + | - | + | + | + |
| CHOP707m | + | + | - | + | - | - |
| U251 MG | + | + | + | - | - | - |

Summary of the data obtained from the indirect immunofluorescence studies of each cell line. The patterns of staining are described as - = no staining, + = positive staining. NF-L, NF-M and NF-H are the low, middle and high molecular weight neurofilament subunits, respectively. The following antibodies were used to obtain these data; nestin (rabbit anti-nestin antiserum 129), vimentin (V9), GFAP (2.2B10), NF-L (NR-4, RMS12), NF-M (RMdO20, RMO254, HO14), NF-H (DP1, TA51, RMO24).

Except for CHOP707m, which was derived from a cerebral neuroblastoma (Baker, D.L. et al., Am. Neurol. 28:136 (1990)), the other 6 PNET cell lines were obtained from cerebellar medulloblastomas (see Hockfield, S. et

al., J. Neurosci 5:3310 (1985)) and citations therein). Daoy does not exhibit any evidence of glial or neuronal differentiation and it was nestin negative, while the other 6 PNET cell lines resembled that of embryonic neuroblasts (He, X. et al., J. Neuropathol. Exp. Neurol. 48:48 (1989)). Double immunofluorescent staining of D283 Med, which is the most differentiated PNET cell line, showed incomplete co-localization of nestin and NF proteins in bundles of IFs in the same cells. Similar observations were made using nestin and vimentin antibodies. CHOP707m expressed extensive nestin immunoreactivity and double immunofluorescence of nestin and vimentin in this cell line co-localized both proteins in the same cells, but the nestin positive filament bundles were located mainly in the perinuclear area while vimentin positive filament bundles were present throughout these cells. The Daoy line expresses vimentin, but not NF proteins, GFAP or other molecular markers of neurons or glia (He, X. et al., J. Neuropathol. Exp. Neurol. 48:48 (1989)). Hence, Daoy is the least differentiated PNET cell line, and it did not express nestin. The foregoing co-localization studies also indicated that the anti-nestin 129 did not cross react with vimentin or NF proteins.

Since nestin is expressed primarily in CNS stem cells and is eliminated during the progressive maturation of the progeny of these stem cells, the Daoy cell line may resemble a CNS precursor that subsequently develops into a vimentin and nestin positive CNS stem cell like those observed here in the human spinal cord at a GA of 6 weeks. Alternatively, tumor cell lines contain genetic

mutations and their phenotype may not fully replicate that of normal cells. However, the 2 glioma cell lines (U251 MG and U373 MG) resembled immature spinal cord radial glia (i.e. at 11 weeks GA) since these cells co-expressed nestin, GFAP and vimentin in the triple fluorescence studies.

Finally, Western blot studies were performed which showed that anti-nestin 129 labeled a single band in cytoskeletal extracts of U251 MG cells, and this immuno-band had a $M_r$ similar to that of the bacterial TrpE-nestin fusion protein and lower than that of rat nestin expressed in P6 rat cerebellum. The significance of the difference in the $M_r$ of rat and human nestin is unclear and will require further studies. Since these extracts contained vimentin, GFAP and other cytoskeletal proteins, these data further demonstrate the specificity of the anti-nestin antiserum for human nestin.

THE EXPRESSION OF NESTIN IN BRAIN TUMOR TISSUE

Brain Tumors Contain Nestin Positive Cells

ST15A cells express properties expected of the precursor to brain tumor cells as described in Example 3. Therefore, brain tumor cells were tested to determine whether they express features in common with ST15A, such as expression of nestin. As described in detail in Example 6, tissue from five medulloblastomas was obtained. Three of these samples came from recurrent tumors occurring in a 16 year old male, a 26 year old male and a 5 year old female. The remaining tumors were

newly diagnosed in an 11 year old male and a 10 year old female. The primary tumor in the 11 year old male was also used as a source of cultured cells.

Immunohistochemistry on sections of tissue from each of the five tumors studied showed regions with large numbers of nestin immunoreactive cells. The expression of nestin in medulloblastoma tissue was not uniform and in some cases parts of the tumor tissue showed a few nestin-positive cells among nestin-negative cells. The pre-immune serum showed no reaction with either human embryonic or tumor tissue. The nestin positive cells were small with a cytoplasmic distribution of the antigen. In one tumor, there were groups of aligned nestin positive cells which were very similar in their morphology to the embryonic neuroepithelium.

A cell line which was generated by growing cells out of the medulloblastoma from the 11 year old male was also tested for expression of nestin. Immunocytochemistry with polyclonal anti-nestin antiserum shows that these cells are uniformly positive for nestin expression and reveals the filamentous pattern characteristic of this intermediate filament protein. Western blotting using anti-nestin serum shows that the antigen detected in these cells comigrates with rat nestin.

Sections of normal fetal and adult human cerebellum were also stained with polyclonal antiserum against nestin. The fetal tissue showed many positive cells in the proliferative, external granular layer of the developing cerebellum and in the outer part of the internal granular layer. In contrast, there was no

immunoreactivity in cerebellum from a 2.5 year old. These results are consistent with the stem cell specific expression of nestin seen in developing mouse and rat brain.

05 <u>Utility</u>

The adult cell types of the brain express distinct intermediate filament proteins: neurofilaments are expressed by neurons, peripherin by a subset of neurons (Leonard, Gorham, <u>et al</u>., (1988)), and glial fibrillary acidic protein by astrocytes. The expression of nestin defines an earlier stage in the pathway of intermediate filament gene expression. Early embryonic cells are nestin negative but express certain cytokeratins (Jackson, B.W., C. Grund <u>et al.</u>, <u>Differentiation</u> <u>20</u>: 203-216 (1980)). During neurulation neurectodermal cells become nestin positive. Upon terminal differentiation, CNS cells stop producing nestin and express class III or IV intermediate filaments typical of their differentiated cell type.

Nestin positive neurectodermal cells have been shown to be capable of differentiating into neuronal, glial and muscle cells (i.e., the different cell types found in tumors). Nestin expression has also been directly detected in tumor tissue and in a medulloblastoma derived cell line. Nestin positive cells derived from tumors are likely to be renewing stem cells which give rise to the differentiated cells in the tumor.

The detection of nestin expression in brain cells obtained from an adult using known methods is indicative of the presence of a brain tumor or of neural precursor

cells capable of developing into a brain tumor. For the purposes of the subject invention, an "adult" is an individual whose central nervous system is developed and, therefore, contains differentiated cells. For example, the central nervous system of human neonates is in continual development up until the age of about one year. Therefore, a human adult, for the purposes of the subject invention, is an individual who is older than about one year.

Nestin mRNA expression in a brain tissue sample can be detected using a DNA probe; this is indicative of expression of nestin, which is, in turn an indication of a predisposition to the development of a brain tumor or of the presence of a brain tumor in the individual. For example, a sample of brain tissue from of an individual can be hybridized to a DNA probe which is complementary to all or a portion of the nestin gene. Detection of hybridization is an indication of a predisposition to the development of a brain tumor or the presence of a brain tumor.

Alternatively, expression of the nestin protein can be detected using polyclonal antibodies, (e.g., anti-nestin antiserum 129) or monoclonal antibodies (e.g., Rat 401). For example, cerebral spinal fluid or a serum sample from the brain of an individual can be stained with anti-nestin antibodies. Detection of stained cells is an indication of a predisposition to the development of a brain tumor or the presence of a brain tumor.

The finding that neural precursors to tumors express nestin also has important therapeutic implications. For example, the nestin marker enables further investigation

into the proliferation and differentiation of the stem cells of a brain tumor, so that existing chemotherapies can be implemented at an earlier stage in the tumor development and so that new methods of inhibiting tumor development can be developed.

The present invention will now be illustrated by the following examples, which are not to be seen as limiting in any way.

## EXEMPLIFICATION

### EXAMPLE 1 Cloning and Characterization of the Rat Nestin Gene

#### Construction and Screening of Libraries

λgt10 and λgt11 cDNA libraries were constructed from oligo-dT-primed poly(A)+ RNA from embryonic day 15 dissected rat CNS according to procedures which were slightly modified from those described (Young, R.A. and R.W.Davis, Proc. Natl. Acad. Sci. USA. 80: 1194-1198 (1983)). The second strand cDNA was synthesized using the RNase H modification of the Okayama-Berg method (Gubler, U. and R. Hoffman, Gene 25: 263-269 (1983)). After induced protein expression, the λgt11 library was screened with the monoclonal antibody Rat 401 (undiluted hybridoma supernatant) and positive clones were visualized with an anti-mouse second antibody conjugated to alkaline phosphatase (Promega Protoblot). Immunopositive clones were selected and the cDNA insert from the clone giving the strongest hybridization signal, λgt11.401:16,

was $^{32}P$-labelled (Feinberg, A.P.and B. Vogelstein, Anal. Biochem. 137: 266-267 (1983) and used as probe to screen the λ gt10 library (Benton W.D.and R.W. Davis, Science. 196: 180-182 (1977); Thomas, P.S. Proc. Natl. Acad. Sci. USA. 77: 5201-5205 (1980)). Several clones were identified. A commercial rat genomic DNA library (Clontech) in the vector Charon 4A was screened with $^{32}P$-labelled nestin cDNA inserts as above. A number of hybridizing clones were obtained, and a genomic map comprising 20 kb of the nestin gene region was established by restriction mapping of the genomic clones, cross-hybridizations, and sequencing of selected genomic subclones.

DNA Sequencing and Computer Analysis

cDNA inserts and genomic subclones were sequenced after subcloning into M13mp18, M13mp19, or Bluescript KS+ (Stratagene). M13 phage particles and Bluescript were grown and circular DNA prepared according to standard procedures. Both single stranded (M13) and double stranded (Bluescript) sequencing was performed using a modified T7 DNA polymerase (Sequenase, USB), as suggested by the manufacturer. Approximately 90% of the gene, including all exons and cDNA inserts, was sequenced on both strands. Ambiguous regions were further resolved using deoxyinosine in the standard Sequenase protocol. Computer analysis was performed using the University of Wisconsin Genetics Computer Group program package, including the FASTP and TFASTA algorithms (Devereux, Haeberli et al., 1984)). The complete sequence, including introns, will be stored in the EMBL Database.

Isolation of RNA and Northern Blotting

Tissue was rapidly dissected and quickly frozen in liquid nitrogen. After homogenization in a quanidinium thiocyanate containing buffer, total RNA was isolated by centrifugation through a CsCl cushion (Chirgwin, Przybyla et al., 1979)). Poly(A)+ RNA was isolated by an oligo-dT chromatography column (Aviv and Leder, 1972) or by a poly(A)+ RNA extraction kit (Fast Trak, Invitrogen). For Northern blot analysis RNA was denatured and electrophoresed in 1% agarose gels containing 2.2 M formaldehyde in 1X MOPS buffer (Maniatis, Fritsch et al., 1982) at +4°C for 8 hours. The RNA was then transferred to nitrocellulose filters (GeneScreen Plus, New England Nuclear) (Thomas, 1980)). The filters were prehybridized and hybridized (50% formamide, 0.75M NaCl, 100 mM Tris pH 7.8, 5 mM EDTA, 1% sodium dodecyl sulphate at 42°C for 16 hours) with 2 x $10^6$ cpm/ml of the $^{32}$P-labeled nestin cDNA insert from λ gt10.401:16 (specific activity 3 x $10^8$ cpm/ug). After hybridization, the filters were washed (final wash = 2 x 30 minutes at 65°C in 30 mM NaCl, 4 mM Tris pH 7.8, 0.2 mM EDTA and 0.25% SDS) and exposed to x-ray film with intensifying screens. Quantity and integrity of the RNA were monitored by rehybridizing the blots with a cDNA probe from the human beta-tublin gene under identical conditions. The size of the hybridizing RNA was determined by running RNA molecules of known size (HMW RNA ladder, BRL) in parallel.

S1 Nuclease Mapping and Primer Extension

To generate a probe for S1 nuclease protection experiments, we amplified a region of a genomic clone

between two synthetic primers using the polymerase chain reaction (kit from Perkin-Elmer Cetus). The resulting fragment was 315 bp long and located between bp -121 and +194 (bp +1 indicates the subsequently defined initiation of transcription). After $^{32}P$ end-labelling, 300 fmole of the fragment was hybridized to 50 ug of E15 CNS rat total RNA or 50 ug of yeast total RNA according to standard procedures, and the S1 resistant hybrids separated on denaturing polyacrylamide gels (Maniatis, T., E.F. Fritsch _et al_., _Molecular cloning: A laboratory manual (Cold Spring Harbor, New York:_ (1982)). $^{32}P$ end-labeled restriction digested plasmids and sequencing reactions of known DNAs were used as size standards. The dried gels were exposed to x-ray film. Primer extensions were performed by hybridizing 125 fmole of one of the oligonucleotides used in the polymerase chain reaction (pos +170 - +194) to 10 ug of poly(A)+ RNA from rat E15 CNS according to standard procedures. The reaction product was subjected to gel electrophoresis as in the S1 nuclease experiments.

_Immunocytochemistry_

For all experiments cells from the immortalized cell line ST15A were grown for 2 days (to 40% confluence) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum on sterile, polyornithine coated glass cover slips, as described in U.S. Patent Applications Serial Nos. 07/603,803; 07/201,762 and 180,548. All antibody incubations lasted 60 minutes at room temperature and were followed by three rinses in PBS (0.14M)

NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$ and 8 mM $KH_2PO_4$ pH 7.2). In the double-lable experiments with nestin and tubulin antibodies the ST15A cells were briefly rinsed in PBS, presoaked in microtubule stabilizing buffer (MTSB = 2M glycerol, 0.1 M PIPES pH 6.9, 1 mM $MgSO_4$, 2 mM EDTA) for 2 minutes and then fixed for 5 minutes in 4% para-formaldehyde, 0.5% NP-40 in MTSB for 5 minutes. After two brief rinses in PBS and 5 minutes incubation in blocking solution (0.75% Triton X-100, 500 ug/ml bovine serum albumin, 1% horse serum and 0.05% sodium azide), the cells were incubated with a polyclonal rabbit-anti-tubulin antiserum (kind gift of Dr. Frank Solomon) diluted 1:10000 in PBS. This was followed by an incubation with a fluorescein-conjugated goat-anti-rabbit IgG antiserum (Cappel, No. 1612-0081) diluted 1:100 in PBS. The cells were then postfixed in 95% ethanol/5% acetic acid for 5 seconds and rinsed three times in PBS. This was followed by an incubation with undiluted hybridoma supernatant from the monoclonal mouse-anti-nestin antibody (Rat 401). Finally, the cells were incubated with a rhodamine-conjugated polyclonal goat-anti-mouse IgG antibody (1:100 dilution, Cappel No. 2611-0231). After two brief rinses in water, the cells were covered with Immumount (Shandon) and inverted onto clean glass microscope slides. As control experiments for potential cross-hybridization and spillover between the fluorescein and rhodamine spectra in the fluorescence microscope, the first antibodies (rabbit-anti-tubulin and mouse-anti-nestin) were omitted in two separate experiments under otherwise identical conditions. No cross-hybridization

or significant spillover was observed. In the colchicine experiments the St15A cells were grown as above and treated with colchicine at 25 ug/ml for 24 hours followed by fixation and staining as described here.

In the actin/nestin double-label experiments ST15A cells were grown as above and fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS for 5 minutes. After blocking as described above, the cells were incubated with 0.16 uM rhodamine-conjugated phalloidin, as suggested by the manufacturer (Molecular Probes). Rinses, postfixation and staining with the nestin monoclonal antibody were performed as described above, with the exception that a fluorescein-conjugated sheep-anti-mouse IgG second antibody (diluted 1:00, Cappel No. 1606-3152) was used. No cross-hybridization or spillover was observed after omitting the rhodamin-conjugated phalloidin or the nestin antibody. In the cytochalasin B experiments the ST15A cells were treated with cytochalasin B at a final concentration of 10 ug/ml for 60 minutes, otherwise all immunostaining procedures were identical.

In the vimentin/nestin double-label experiments ST15A cells were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS for 5 minutes. After blocking, the cells were incubated with a polyclonal goat-anti-vimentin antiserum (dilution 1:20, MILES 65-794) followed by a fluorescein-conjugated rabbit-anti-goat IgG second antibody (dilution 1:100, ICN 65-176). Incubation times and rinsing procedures were as described above. Postfixation and incubation with the anti-nestin antibody

were performed as in the tubulin/nestin experiments. No cross-hybridization or spillover was detected after omitting the anti-vimentin and anti-nestin antibodies, respectively. In all experiments the mounted cells were photographed on Kodak Ectachrome 400 ASA using a 63X objective on a Zeiss fluorescence microscope.

EXAMPLE 2 Production of the Rat 401 Monoclonal Antibody

Developing monoclonal antibodies were generated to fixed spinal cord from embryonic day 15 (E15) rats. Timed pregnant female rats were obtained from Taconic Animal Supply Co. and housed until the appropriate gestation age. Uteri were dissected from pregnant animals into ice-cold phosphate buffer (pH 7.4), individual enbryos were removed and the spinal cord was dissected free of other tissue into fixative (4% paraformaldehyde in 0.1 M phosphate buffer).

The immunization and fusion protocol has been described previously (McKay and Hockfield, Proc. Natl. Acad. Sci. USA 79: 6747-6751 (1982)). Briefly, BALB/c mice received two intraperitoneal immunizations with fixed tissue homogenized in saline and suspended in an equal volume of Freud's complete adjuvant and a final intravenous boost of unfixed tissue with adjuvant. Spleen cells from immunized mice were fused with NS1 myeloma cells and resulting hybrid cell lines were screened immunohistochemically on 100 μm-thick Vibratome sections of 4% paraformaldehyde-fixed E15 rats. Lines producing antibodies of interest were cloned in soft agar or by limiting dilution and antibody subclass was determined by the Ouchterlony method (Ouchterlony and Nilsson,

Immunodiffusion and Immunoelectrophoresis. In Handbook of Experimental Immunology D.M. Weir, ed., Blackwell Scientific Publications, Oxford (1978).

Immunohistochemistry

Tissue for immunohistochemistry was fixed either by intravascular perfusion or by immersion. All postnatal material was fixed by intravascular perfusion (under ether anesthesia) with 4% paraformaldehyde at pH 7.4 for light microscopy or with either 4% paraformaldehyde at pH 10.0 or 4% paraformaldehyde, 0.1% glutaraldehyde at pH 7.4 for electron microscopy. The CNS was dissected out and stored in 0.1 m phosphate buffer (pH 7.4) with 0.1% sodium azide. For prenatal material, pregnant females were ether anesthetized, uteri were dissected into cold 0.1 m phosphate buffer and the females were killed by cervical dislocation. Embryos were dissected individually into Sylgard-coated Petri dishes and pinned in place. Intravascular perfusion was performed by opening the skin of the thoracic cavity, nicking the right atrium with a fine forceps and placing a 25 gauge needle into the left ventricle. One to 5 ml of fixative (as above) were injected into the heart. The embryos were placed in a vial containing the same fixative for 4 hr and then stored in phosphate buffer. E12 to term embryos were fixed by perfusion (using a 30 gauge needle for E12 embryos); E10 and E11 embryos were fixed by immersion in fixative. E13 and older animals were sectioned at 50 to 100 $\mu$m on a Vibratome without additional support. E10 to E12 embryos were embedded in 15% gelatin before sectioning.

Immunohistochemical reactions were performed on free-floating sections by sequential incubations in monoclonal antibody as full-strength supernatant (12 to 20 hr); horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody at a dilution of 1:100 in tissue culture medium with 10% serum; and 3.3'-diaminobenzidine (DAB; 0.025%) with $H_2O_2$ (0.002%). Peroxidase-conjugated lectins (Sigma Chemical Co.) were used at serial dilutions in the same manner with the omission of the peroxidase-conjugated goat anti-mouse antibody. For light microscopy, 2% Triton X-100 was included in both antibody solutions. For electron microscopy sections carried through this procedure (without Triton X-100) were post-fixed in 2% $O_2O_4$ embedded in Epon/Araldite between plastic coverslips, and cut at 1 μm and 0.12 μm for correlative light and electron microscopy. For high resolution light microscopy, sections (Triton-treated) were embedded in plastic as for electron microscopy and sectioned at 2 μm.

Immunoblots

The apparent molecular weights of antigens recognized by antibodies were determined using the method of Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350-4354 (1979)). Proteins were extracted from unfixed tissue with sodium dodecyl sulfate (SDS) and β-mercaptoethanol and run on an SDS-polyacrylamide gel. The proteins were electroblotted onto nitrocellulose paper which was then exposed to primary (1 hr) and secondary (1 hr) antibodies. HRP was visualized with 4-chloronaphthol.

EXAMPLE 3 Chacterization of Rat Nestin from the ST15A Cell Line

ST15A cells were derived from rat postnatal day 2 cerebellum after infection of a primary culture with a retrovirus transducing the temperature sensitive, tsA58 allele of SV40 T antigen (Frederiksen et al., 1988)). ST15A cells were plated on tissue culture plastic coated with polyornithine and passaged at the permissive temperature, 33°C, in Dulbecco's modified Eagle's medium (DME) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 15 mM HEPES, pH 7.2, containing 10% fetal calf serum (FCS). For the experiment in Figure 8, confluent cells (labelled 'day 0') were transferred into DME/10% horse serum (HS) and cultured at 33°C or 39°C as marked for the indicated time. 39°C is the non-permissive temperature for tsA58.

Primary cultures were prepared from P4 rat cerebellum as previously described (McKay, R.D.G. et al., Cold Spring Harbor Symp. Quant. Biol. (1990). Briefly, tissue was dissociated by trypsinization followed by trituration. Cells were plated on polyornithine-coated coverslips in DME/10% FCS/10% HS and grown at 39°C for 24 hours. The medium was changed to DME/10% HS and culture continued for 6 days.

The medulloblastoma cell line was derived by growing cells from dispersed, fresh medulloblastoma tissue. The tissue was removed from a previously untreated tumor in a 10 year old female. The cell line has not been cloned. Cells were passaged in Ham's F12 supplemented with 20mm L-glutamine and 10% FCS.

Immunocytochemistry

Cells for immunocytochemistry were fixed in 4% paraformaldehyde buffered with 50 mM sodium phosphate, pH 7.2, for troponin staining or buffered with 50mM sodium borate, pH 9.5, for nestin staining. The antibodies used were mouse monoclonal Rat 401 (Hockfield and McKay, supra (1985), mouse monoclonal antibody against troponin T (Sigma T-6277), and rabbit polyclonal serum raised against the C-terminal region of nestin expressed in bacteria. Fixed cells were incubated in primary antibody for one hour, rinsed 3 times in PBS, incubated for one hour with flourescein-conjugated goat anti-rabbit serum (Organon-Tetnika) and mounted in Immumount (Shandon, Inc.) with 2% DABCO. Medulloblastoma tissue and normal human cerebellum were frozen and subsequently fixed in formalin. 50 um sections were cut and stained with rabbit polyclonal serum raised against the C-terminal region of nestin.

Isolation of RNA and Northern blotting

Cultured cells were scraped into 5M guanidine thiocyanate/25 mM citrate, pH 6.8/ 0.5% N-lauryl sarcosine/0.1 $\beta$-mercaptoethanol and RNA pelleted through a CsCl step gradient (Chirgwin et al., 1979). 20 ug of total RNA was electrophoresed through a 1% agarose/2.2M formaldehyde gel and transferred to nitrocellulose. Prehybridization and hybridization were in 50% formamide/5xSSC/5X Denhardt's/0.1% SDS/0.1 mg/ml salmon sperm DNA at 42$^{o}$C. Filters were washed in 2xSSC/0.1% SDS at room temperature and in 0.1xSSC/0.1% SDS at 55$^{o}$C and

exposed to X-ray film with an intensifying screen. Filters were stripped by washing 6 times for 3 minutes each in boiling 0.05xSSC/0.1% SDS/20 mM EDTA, pH8, and rehybridized. Probes were prepared for hybridization by random priming on fragments isolated on low melting temperature agarose gels (Feinberg and Vogelstein, supra (1984). The following plasmids were used to generate probes: mouse myoD1 cDNA pEMC11s (Davis, R.L. et al., Cell. 51: 987-1000 (1987); mouse myogenin cDNA p65 (Wright et al., supra (1989); rat nestin cDNA p401-16; human B-actin cDNA (Gunning, P. et al., Mol. Cell. Biol. 3: 787-795 (1983)).

Western Blotting

Proteins were extracted from cultured cells or rat postnatal day 5 limb muscle (Sprague-Dawley; Taconic, Inc.) in the presence of aprotinin and PMSF in 2% SDS, 5 mM EDTA and 15% glycerol in 0.1 M Tris, pH 6.8. 50 ug of total protein was run in each lane of an SDS reducing gel according to Laemmli (Laemmli 1970); proteins were transferred to Immobilon P membranes (Millipore) using a Hoeffer transfer appartus at 50 volts for 4 hours. Filters were blocked in 5% BLOTTO (Johnson, D.A. et al., Gene Anal. Tech. 1: 3-8 (1984)) overnight. Rat 401 hybridoma supernatant was used undiluted; troponin T antibody was diluted 1:500; polyclonal anti-nestin antiserum was diluted 1:1000; HRP conjugated secondary antibodies were purchased from BioRad.

EXAMPLE 4 Characterization of Human Nestin

To characterize human nestin, a new antiserum, designated anti-nestin 129, was raised to recombinant rat nestin expressed in E. coli since the rat-401 mAb failed to recognize human nestin in immunohistochemical and immunochemical assays.

Production of Anti-nestin Antiserum

The insert from the clone λgt10 401:16 (Lendahl, U. et al., Cell 60: 585 (1990)) was isolated and ligated into the pATH1 vector (Tzagaloff, A. et al., J. Biol. Chem. 262: 17163 (1986)), producing a clone in which the bacterial TrpE protein (37.2 kD) was fused at its C terminus with the last 1197 amino acids of nestin. The fusion protein was induced in E. coli strain HB101 with 20 µg/ml indoleacrylic acid (Sigma I1625) for 4 hours as described in (Tapscott, S. et al., Science 242: 405 (1988)). The TrpE-nestin fusion protein migrates at greater than 200 kD in 6% SDS PAGE gels, making it the largest protein in the bacterial lysate. The fusion protein was purified by cutting the top band from the gel. Anti-nestin antiserum 129 was produced by injecting the fusion protein subcutaneously into a female NZW rabbit. The characterization of anti-nestin 129 and its specificity for human nestin is described here.

First, this antiserum was compared to the Rat-401 MAb in immunochemical and immunohistochemical studies of postnatal day 6 (P6) rat cerebellum. Then, anti-nestin 129 was used to probe human CNS tissues, tumors and tumor derived cell lines. To determine if nestin co-localized with other IF proteins, double and triple immuno-

fluorescence experiments also were performed using anti-nestin 129 and antibodies to vimentin, glial fibrillary acidic protein (GFAP) and neurofilament (NF) triplet proteins. Finally, Western blots were performed on cytoskeletal extracts of some of the tumor derived cell lines to identify human nestin and to compare it with rat nestin as well as with the class III and IV IF proteins expressed by CNS cells.

Specificity of Anti-Nestin 129 in the Rat

The rabbit anti-nestin antiserum produced as described herein, i.e. anit-nestin 129, yielded results identical to those produced by the Rat-401 MAb when both were used to probe P6 and adult rat CNS tissues by immunoblots and immunohistochemistry. On immunoblots of cytoskeletal extracts from P6 rat cerebellum separated by SDS-PAGE, anti-nestin 129 identified a band with an apparent $M_r$ of 240 kD. This band was identical to the band labeled by Rat-401. Further, both antibodies labeled similar bands in lysates of the induced bacteria producing the TrpE-nestin fusion protein.

In immunohistochemical studies of the P6 rat cerebellum, anti-nestin 129 labelled radial glial cells very intensively. The processes of these cells radiated from the internal granular layer to the external granular layer. These radial glia also were labeled with Rat-401 and the anti-vimentin MAb. Neither of the anti-nestin antibodies stained immature Purkinje cells or other neurons. In contrast to the P6 rat cerebellum, the anti-nestin 129 and Rat-401 antibodies only stained blood vessel endothelial cells in the adult rat cerebellum,

while the anti-vimentin MAb (V9) labelled blood vessels, white matter astrocytes and radial glial fibers. Furthermore, nestin immunoreactivity was demonstrated by both anti-nestin antibodies in immature skeletal muscle of the P6 rat as well as in Schwann cells of adult rats (data not shown).

These studies demonstrate that this new anti-nestin antiserum yields immunohistochemical results in the human developing spinal cord, cerebrum and cerebellum that were nearly identical to those produced by Rat-401 and the anti-nestin antiserum in the rat.

EXAMPLE 5 Cloning of the Human Nestin Gene

The human nestin gene was isolated using low-stringency DNA hybridization of a human genomic bacteriophage lambda library with a rat nestin probe.

A genomic library in the vector EMBL 3 made from partially Sau3A-digested human genomic DNA (Clontech) was screened using a $^{32}$P-labelled probe derived from the rat nestin gene starting at 202 nucleotides upstream of the translation start and ending at nucleotide 483 after the translation start (Lendahl, et al., 1990) under the following conditions: prehybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 micrograms/ml salmon sperm DNA at 65°C for 1 hour; hybridization in the same conditions except for using 50 micrograms/ml salmon sperm DNA and hybridizing for 16 hours. The filters were then washed for 2 X 30 minutes at 48°C in 2X SSC and 0.25% SDS.

One positive clone out of 1.5 X $10^6$ placques screened contained the entire human nestin gene and was

chosen for further analysis. This clone was purified and grown in large scale (Maniatis, et al., 1989). A restriction map was established and EcoRI fragments were subcloned in the plasmid vector Bluescript KS I (Stratagene).

EXAMPLE 6 Nestin Expression in Brain Tumor Samples

Immunohistochemical data on the expression of nestin, other IF proteins and synaptophysin (SYP) by a diverse group of 34 CNS tumors are summarized in Table 2.

TABLE 2 Immunoperoxidase staining of brain tumors

| No. | Age(yr) | Sex | Material | Location | Nestin | Vim | GFAP | NP | SYP |
|---|---|---|---|---|---|---|---|---|---|
| PNETs | | | | | | | | | |
| PNET+N,G | | | | | | | | | |
| 1 | 1 | M | F | posterior fossa | + | +++ | + | + | - |
| 2 | 1 | M | F | posterior fossa | + | +++ | + | +++ | +++ |
| 3 | 5 | F | F | posterior fossa | ++ | + | + | + | ++ |
| 4 | -1 | M | F | posterior fossa | ++ | ++ | + | + | +++ |
| 5 | 1 month | F | F | posterior fossa | ++ | ++ | + | +++ | + |
| 6 | 5 | M | F | posterior fossa | ++ | ++++ | ++ | + | + |
| 7 | 8 months | F | F | posterior fossa | +++ | +++ | ++ | + | + |
| 8 | 1 | F | F+P | posterior fossa[a] | ++ | ++ | + | + | +++ |
| PNET+N | | | | | | | | | |
| 9 | 13 | F | F | posterior fossa[b] | - | ++++ | - | ++ | ++ |
| 10 | 6 | M | F | posterior fossa | - | + | E | ++ | ++ |
| PNET+G | | | | | | | | | |
| 11 | 11 | M | F | posterior fossa | + | +++ | ++ | P | + |
| 12 | 4 | F | F | posterior fossa | +++ | ++++ | ++ | P | +++ |
| PNET,NOS | | | | | | | | | |
| 13 | 2 | M | F | pineal | ++ | ++ | - | - | ++ |
| 14 | 11 | M | F | posterior fossa[b] | - | + | - | - | ++ |
| 15 | 14 | M | F | posterior fossa[b] | + | +++ | - | - | + |
| Astrocytic tumors | | | | | | | | | |
| 16 | 2 | F | F | posterior fossa[c] | ++ | ++++ | ++++ | - | - |
| 17 | 6 | F | F+P | posterior fossa[c] | - | ++++ | +++ | - | |
| 18 | 6 months | M | F | hypothalamus[c] | ++ | +++ | +++ | - | - |
| 19 | 17 | M | F+P | posterior fossa[d] | - | +++ | +++ | - | - |
| 20 | 3 | M | F | frontal[e] | +++ | NA | + | - | NA |
| 21 | 6 | M | F+P | occipital[f] | +++ | +++ | +++ | - | - |
| 22 | 13 | M | F | posterior fossa[f] | +++ | +++ | +++ | - | - |
| Ependymomas | | | | | | | | | |
| 23 | 3 | F | F | parietal | ++ | ++++ | + | - | - |
| 24 | 2 | M | F | posterior fossa | + | - | + | - | - |
| 25 | 3 | M | F+P | posterior fossa | ++ | ++++ | +++ | - | - |
| 26 | 5 | M | F+P | posterior fossa | ++ | ++++ | ++ | - | - |
| 27 | 15 | F | F+P | cerebral hemisphere[g] | ++ | ++++ | + | - | - |
| 28 | 8 | F | F+P | parietal[h] | +++ | +++ | ++ | - | - |
| Choroid plexus papillomas | | | | | | | | | |
| 29 | 1 | M | F | posterior fossa | - | +++ | + | - | - |
| 30 | 2 | M | F | lateral ventricle[i] | - | +++ | - | - | - |
| Gangliogliomas | | | | | | | | | |
| 31 | 2 | F | F | temporal | ++ | +++ | +++ | + | - |
| 32 | 1 | F | F | occipital | ++ | ++ | +++ | + | - |
| Meningiomas | | | | | | | | | |
| 33 | 8 | M | F | posterior fossa | ++ | ++ | - | - | - |
| 34 | 27 | M | F+P | anterior fossa | + | +++ | - | - | - |

Legend to Table 2

Summary of the data obtained from the immunoperoxidase studies of each of the brain tumor samples. Results from frozen (F) and paraffin (P) material are combined in the cases in which both kinds of material were studied. The patterns of staining are described as - = no staining, + = positive in less than 5% of tumor cells, ++ = positive in between 5 - 50% of tumor cells, +++ = positive in between 50 - 95% of tumor cells, ++++ = positive in more than 95% of tumor cells, E = positive cells equivocal, i.e. reactive astrocytes of tumor cells, P = interstitial filamentous staining in the neuropil. The following antibodies were used to obtain these data; nestin (rabbit anti-nestin antiserum 129), vimentin (V9), GFAP (2.2B10), NF (RMS12, RMd020, RM0254, HO14, DP1, TA51, RM024). NA = not available due to limited material; PNET+N,G = PNET with neuronal and glial differentation; PNET+N = PNET with neuronal differentiation; PNET + G = PNET with glial differentiation; PNET-NOS = PNET, not otherwise specified. [a]reccurent tumor of case 7, [b]autopsy case, [c]benighn astrocytoma [d]genighn strocytoma + angioma, [e]anaplastic astrocytoma [f]glioblastoma multiforme, [g]mixed ependymo-astrocytoma, [h]anaplastic enpendymoma, [i]choroid plexus carcinoma.

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The results from frozen and ethanol-fixed paraffin-embedded samples from the same biopsy are combined in the 10 cases from which these paired samples were available. Notably, the immunohistochemical results obtained from each of these paired samples were identical, although the primary antibodies were used at slightly higher dilutions in the frozen material compared with the paraffin material.

PNETs The anti-nestin antiserum stained tumor cells in 12 of the 15 PNETS examined here. Nestin immunoreactivity was found in the cytoplasm of individual neoplastic cells. Reactivity was also observed in the coarse processes of large stellate cells, as well as in

blood vessel endothelial cells. In the PNETs with an insular architecture, nestin reactive cells were found both within and outside the islands. Since PNETs express SYP and other neuroendocrine markers as well as all classes of IF proteins (Gould, V.E. et al., Human Pathol. 21:245 (1990)), the PNETs were probed for SYP. These studies demonstrated SYP in 14 of 15 PNETs. Vimentin was detected in all the PNETs. Ten of the 15 PNETs expressed GFAP and 10 expressed 1 or more NF proteins. Double immunofluorescence studies of nestin and NF proteins, as well as nestin and GFAP on frozen sections showed their co-localization in the same cells (data not shown). The immunohistochemical data on GFAP and NF proteins suggested that 15 PNETs could be classified into 4 subtypes, i.e. 3 cases of PNET-NOS (not otherwise specified), 2 cases of PNET+G (PNET with glial differentiation), 2 cases of PNET+N (PNET with neuronal differentiation) and 8 cases of PNET+G,N (PNET with glial and neuronal differentiation). All 8 cases of PNET+G,N showed nestin immunoreactivity and 2 cases of PNET+G expressed nestin. On the other hand, 2 cases of PNET+N were negative for nestin. These results may indicate that PNETs with neuronal differentiation tend to lack nestin expression in contrast to PNETs with glial differentiation. This phenomenon paralleled normal differentiation in that postmitotic neuroblasts did not express nestin whereas radial glial cells did, even at relatively late developmental stages. Two of 3 cases of PNET-NOS showed nestin positive tumor cells. This immunohistochemical phenotype might represent primitive neuroepithelial cells or immature radial glial cells in the

developing CNS. The other PNET-NOS case was negative for nestin and it might correspond to extremely immature CNS precursor cells just like the most undifferentiated PNET cell line (i.e. Daoy).

<u>Astrocytic tumors</u> Five of 7 astrocytic tumors displayed positivity for nestin in the perinuclear cytoplasm of the tumor cells as well as in the cytoplasmic processes of these cells. Additionally blood vessel walls were nestin positive. Two of 4 benign astrocytomas were negative for nestin. One anaplastic astrocytoma and 2 glioblastomas showed strong nestin positivity. In the glioblastomas, multinuclear giant cells were stained strongly.

<u>Ependymomas</u> Six tumors were histologically diagnosed as ependymomas and all showed nestin positivity. Immunoreactivity for nestin was found in the tumor cells which formed perivascular pseudo-rosetts. One ependymoma (with marked nuclear pleomorphism, necrosis and a few mitoses) was diagnosed as an anaplastic ependymoma and it demonstrated the strongest nestin immunoreactivity among the 6 ependymomas. Expression of nestin described here indicates that nestin is the dominant IF protein of early CNS precursor cells. However, nestin is rapidly extinguished in neurons, glia and other CNS cell types derived from these precursors. The mechanisms responsible for these rapid changes in IF protein expression are unknown.

In contrast to the restricted expression of nestin in normal developing CNS cells, this IF protein was ubiquitously present in a wide variety of CNS neoplasms.

Nestin was expressed in a subset of PNETs, but was more abundant in gliomas. The presence of nestin in diverse types of neuroepithelial tumors is similar to vimentin, but distinct from the distribution of GFAP and NF proteins in CNS tumors.

## CLAIMS

1. Isolated DNA encoding a protein of mammalian origin whose expression distinguishes neural multipotential stem cells from neuronal, glial and muscle cells.

2. Isolated DNA of Claim 1 wherein the protein is nestin.

3. Isolated DNA of Claim 2 having the nucleotide sequence of all or a portion of the nucleotide sequence of Figure 1 or Figure 2.

4. Isolated DNA having a nucleotide sequence corresponding to all or a portion of the nucleotide sequence of Figure 1.

5. Isolated DNA having a nucleotide sequence corresponding to all or a portion of the nucleotide sequence of Figure 2.

6. Isolated DNA which hybridizes to all or a portion of the DNA sequence represented in Figure 1.

7. Isolated DNA which hybridizes to all or a portion of the DNA sequence represented in Figure 2.

8. Isolated DNA having a nucleotide sequence corresponding to all or a portion of the nucleotide sequence of Figure 1 which encodes a protein of mammalian origin whose expression distinguishes neural multipotential stem cells from neuronal, glial and muscle cells.

9. Isolated DNA having a nucleotide sequence corresponding to all or a portion of the nucleotide sequence ofFigure 2 which encodes a protein of mammalian origin whose expression distinguishes neural multipotential stem cells from neuronal, glial and muscle cells.

10. A DNA probe for the detection of a brain tumor, the probe comprising DNA which hybridizes to all or a portion of the DNA sequence represented in Figure 1 or to all or a portion of a DNA sequence sufficiently homologous to the DNA sequence of Figure 1 that it encodes a protein which is indicative of a predisposition to the development of a brain tumor in an individual.

11. A DNA probe for the detection of a brain tumor, the probe comprising DNA which hybridizes to all or a portion of the DNA sequence represented in Figure 2 or to all or a portion of a DNA sequence sufficiently homologous to the DNA sequence of Figure 2 that it encodes a protein which is indicative of a predisposition to the development of a brain tumor in an individual.

12. A method of diagnosing a predisposition to the development of a brain tumor or the presence of a brain tumor in an individual, comprising:
    a) obtaining a sample from the brain of an individual;
    b) treating the sample in a manner that renders DNA present in the sample available for hybridization with a complementary DNA sequence thereby producing a treated sample;
    c) contacting the treated sample with at least one probe which is a DNA sequence which hybridizes with all or a portion of a DNA sequence represented in Figure 1 or Figure 2; and
    d) detecting the hybridization of DNA from the sample with the DNA probe; wherein hybridization is an indication of a predisposition to the development of a brain tumor or the presence of a brain tumor in the individual.

13. A method of diagnosing a predisposition to the development of a brain tumor or the presence of a brain tumor in an individual, comprising:
    a) obtaining a sample from the brain of an individual;
    b) treating the sample in a manner that renders the cells available for staining with a nestin-specific antibody thereby producing a treated sample;
    c) staining the cells of the treated sample with labelled anti-nestin antibodies; and
    d) detecting stained cells as an indication of a predisposition to the development of brain tumors or the presence of a brain tumor in an individual.

14. A method of Claim 13 wherein the antibodies are labelled with a label selected from the group consisting of fluorescein, rhodamine and peroxidase.

15. A method of Claim 13 wherein the nestin-specific antibody is a polyclonal antibody.

16. A method of Claim 15 wherein the polyclonal antibody is anti-nestin 129.

17. A kit for diagnosing a brain tumor, comprising at least one nucleic acid probe, which hybridizes with a DNA sequence represented in Figure 1 or Figure 2.

18. A kit for diagnosing a brain tumor, comprising a first antibody which is nestin-specific and a second antibody which binds to the first antibody and is labelled.

19. A nestin-specific antibody or a pharmaceutical composition comprising a nestin-specific antibody formulated in a solution for parenteral administration for use in _in vivo_ diagnosis, e.g., tumor imaging.

20. Use of a nestin-specific antibody for the manufacture of a diagnostic agent for _in vivo_ tumor imaging.

# Figure 1

1 [illegible] 120

121 [illegible] 240

241 [illegible] 360

361 [illegible] 480

481 [illegible] 600

601 [illegible] 720

721 [illegible] 840

domain 1

841 [illegible] 960

domain 2

961 [illegible] 1080

domain 3

1081 [illegible] 1200

1201 [illegible] 1320

1321 [illegible] 1440

1441 [illegible] 1560

1561 [illegible] 1680

1681 [illegible] 1800

1801 [illegible] 1920

1921 [illegible] 2040

2041 [illegible] 2160

2161 [illegible] 2280

2281 [illegible] 2400

2401 [illegible] 2520

2521 [illegible] 2640

2641 [illegible] 2760

2761 [illegible] 2880

2881 [illegible] 3000

3001 [illegible] 3120

3121 [illegible] 3240

**Figure 1 (Cont'd)**

3241 [illegible] 3360
[illegible]
3361 [illegible] 3480
[illegible]
3481 [illegible] 3600
[illegible]
3601 [illegible] 3720
[illegible]
3721 [illegible] 3840
[illegible]
3841 [illegible] 3960
[illegible]
3961 [illegible] 4080
[illegible]
4081 [illegible] 4200
[illegible]
4201 [illegible] 4320
[illegible]
4321 [illegible] 4440
[illegible]
4441 [illegible] 4560
[illegible]
4561 [illegible] 4680
[illegible]
4681 [illegible] 4800
[illegible]
4801 [illegible] 4920
[illegible]
4921 [illegible] 5040
[illegible]
5041 [illegible] 5160
[illegible]
5161 [illegible] 5280
[illegible]
5281 [illegible] 5400
[illegible]
5401 [illegible] 5520
[illegible]
5521 [illegible] 5640
[illegible]
5641 [illegible] 5760
5761 [illegible] 5880
5881 [illegible] 5940

FIGURE 2

```
   1  ATGGAGGGCT GCATGGGGGA GGAGTCGTTT CAGATGTGGG AGCTCAATCG
  51  GCGCCTGGAG GCCTACCTGG GCCGGGTCAA GGCGCTGGAG GAGCAGAATG
 101  AGCTGCTCAG CGCCGGACTC GGGGGGCTCC GGCGACAATC CGCGGACACC
 151  TCCTGGCGGG CGCATGCCGA CGACGAGCTG GCGGCCCTGC GTGCGCTCGT
 201  TGACCAACGC TGGCGGGAGA AGCACGCGGC CGAGGTGGCG CGCGACAACC
 251  TGGCTGAAGA GCTGGAGGGC GTGGCAGGCC GATGCGAGCA GCTGCGGCTG
 301  GCCCGGGAGC GGACGACGGA GGAGGTAGCC CGCAACCGGC GCGCCGTCGA
 351  GGCAGAGAAA TGCGCCCGGG CCTGGCTGAG TAGCCAGGGG GCAGAGCTGG
 401  AGCGcGAGcT AGAGGCTcTA CGCGTgGCGC ACGAGGAGGA GCGCGTCGGT
 451  CTGAACGCGC AGgCtgCCTG TGCCCCCCGc cTGCCCgcGc cgcCCCgGcc
 501  tcccgcgCCG GCCCCGGAGG tAGAGGAGcT GGcAAGGCGA CTGGGCGAGG
 551  CGTGGCGCGG GGCAGTGCGC GGCTACCAGG AGCGCGTGGC ACACATGGAG
 601  ACGTCGCTGG ACCAGAcCCG CGAGCGCCTG GCCCGGGCGG TGCAGGGTGC
 651  CCGCGAGGTC CGCCTGGAGC TACAGCAGCT CCAGGCTGAG CGCGGAGGCC
 701  TCCTGGAGCG CAGGGCAGCG TTGGAACAGA GGTTGGAGGG CCGCTGgcag
 751  GaGcGGcTGC GGGCtACTGA AAAGTTCCAG CTGGCTGTGG AGGCCCTGGA
 801  GCAGGAGAAA CAGGGCCTAC AGAGCCAGAT CGCTCAGGTC CTGGAAGGTC
 851  GGCAGCAGCT GGCGCACCTC AAGATGTCCC TCAGCCTGGA GGTGGCCACG
 901  TACAGGACCC TCCTGGAGGC TGAGAACTCC CGGCTGCAAA CACCTGGCGG
 951  TGGCTCCAAG ACTTCCCTCA GCTTTCAGGA CCCCAAGCTG GAGCTGCAAT
1001  TCCCTAGGAC CCCAGAGGGC CGGCGTCTTG GATCTTTGCT CCCAGTCCTG
1051  AGCCCAACTT CCCTCCCCTC ACCCTTGCCT GCTACCCTTG AGACACCTGT
1101  GCCAGCCTTT CTTAAGAACC AAGAATTCCT CCAGGCCCGT ACCCCTACCT
1151  TGGCCAGCAC CCCCATCCCC CCCACACCTC AGGCACCCTC TCCTGCTGTA
1201  GATGCAGAGA TCAGAGCCCA GGATGCTCCT CTCTCTCTGC TCCAGACACA
1251  GGGTGGGAGG AAACAGGCTC CAGAGCCCCT GCGGGCTGAA GCCAGGGTGG
1301  CCATTCCTGC CAGCGTCCTG CCTGGACCAG AGGAGCCTGG GGGCCAGCGG
1351  CAAGAGGCCA GTACAGGCCA GTCCCCAGAG GACCATGCCT CCTTGGCACC
1401  ACCCCTCAGC CCTGACCACT CCAGTTTAGA GGCTAAGGAT GGAGAATCCG
1451  GTGGGTCTAG AGTGTTCAGC ATATGCCGAG GGGAAGGTGA AGGGCAAATC
```

FIGURE 2 (Continued)

```
1501 TGGGGGTTGG TAGAGAAAGA AACAGCCATA GAGGGCAAAG TGGTAAGCAG
1551 CTTGCAGCAG GAAATATGGG AAGAAGAGGA TCTAAACAGG AAGGAAATCC
1601 AGGACTCCCA GGTTCCTTTG GAAAAAGAAA CCCTGAAGTC TCTGGGAGAG
1651 GAGATTCAAG AGTCACTGAA GACTCTGGAA AACCAGAGCC ATGAGACACT
1701 AGAAAGGGAG AATCAAGAAT GTCCGAGGTC TTTAGAAGAA GACTTAGAAA
1751 CACTAAAAAG TCTAGAAAAG GAAAATAAAA GAGCTATTAA AGGATGTGGA
1801 GGTAGTGAGA CCTCTAGAAA AAGAGGCTGT AGGCAACTTA AGCCTACAGG
1851 AAAAGAGGAC ACACAGACAT TGCAATCCCT GCAAAAGGAG AATCAAGAAC
1901 TAATGAAATC TCTTGAAGGT AATCTAGAGA CATTTTTATT TCCAGGAACG
1951 GAAAATCAAG AATTAGTAAG TTCTCTGCAA GAGAACTTAG AGTCATTGAC
2001 AGCTCTGGAA AAGGAGAATC AAGAGCCACT GAGATCTCCA GAAGTAGGGG
2051 ATGAGGAGGC ACTGAGACCT CTGACAAAGG AGAATCAGGA ACCCCTGAGG
2101 TCTCTTGAAG ATGAGAACAA AGAGGCCTTT AGATCTCTAG AAAAAGAGAA
2151 CCAGGAGCCA CTGAAGACTC TAGAAGAAGA GGACCAGAGT ATTGTGAGAC
2201 CTCTAGAAAC AGAGAATCAC AAATCACTGA GGTCTTTAGA AGAACAGGAC
2251 CAAGAGACAT TGAGAACTCT TGAAAAAGAG ACTCAACAGC GACGGAGGTC
2301 TCTAGGGGAA CAGGATCAGA TGACATTAAG ACCCCCAGAA AAAGTGGATC
2351 TAGAACCACT GAAGTCTCTT GACCAGGAGA TAGCTAGACC TCTTGAAAAT
2401 GAGAATCAAG AGTTCTTAAA GTCACTCAAA GAAGAGAGCG TAGAGGCAGT
2451 AAAATCTTTA GAAACAGAGA TCCTAGAATC ACTGAAGTCT GCGGGACAAG
2501 AGAACCTGGA AACACTGAAA TCTCCAGAAA CTCAAGCACC ACTGTGGACT
2551 CCAGAAGAAA TAAATAAATC AGGGGGCAAT GAATCCTCTA GAAAAGGAAA
2601 TTCAAGAACC ACTGGAGTCT GTGGAAGTGA ACCAAGAGAC ATTCAGACTC
2651 CTGGAAGAGG AGAATCAGGA ATCATTGAGA TCTCTGGGAG CATGGAACCT
2701 GGAGAATTTG AGATCTCCAG AGGAGTAGAC AAGGAAAGTC AAAGGAATCT
2751 GGAAGAGGAA GAGAACCTGG GAAAGGGAGA GTACCAAGAG TCACTGAGGT
2801 CTCTGGAGGA GGAGGGACAG GAGCTGCCGC AGTCTGCAGA TGTGCAGAGG
2851 TGGGAAGATA CGGTGGAGAA GGACCAAGAA CTGGCTCAGG AAAGCCCTCC
```

FIGURE 2 (Continued)

2901 TGGGATGGCT GGAGTGGAAA ATAaGgatga Ggcagagctg AatctAaGGg
2951 agcaGgatGG CTTCACTGGG AAGGAGGAGG TGGTAGAGCA GGGAGAGCTG
3001 AATGCCACAG AGGAGGTCTG GTTCCCAGGC GAGGGGCACC CAGAGAACCC
3051 TGAGCCCAAA GAGCAGAGAG GCCTGGTTGA GGGAGCCAGT gTGAAGGGAg
3101 GGgctgagGg cctCcaggac CCtgaAgGGC AATCACAACA GGTGGGGAcC
3151 CCAGGcCTCC AGGCTCCCCA GGGgctgCca gaGgcgatag agCCcctGgt
3201 GgAagatgaT GTGGCCcCAG GGGGTGACca AGCCTCCCCA GAGGTCATGT
3251 TGGGGTCAGA GCCTGCCATG GGTGAGTCTG CTGCGGGagC TGAGCCAGgc
3301 ctGGGgcaGG GGgtGGgaGG gCtGGGGgaC CcaggCcATC TgACCAGGGA
3351 AGAGGTGATG GAACCACCCC TGGAAGAGGA GAGTTTGGAG GCAAAGAGGG
3401 TTCAgGGCTT GGAAGGGCCT AGAAAGGACC TAGAGGAGGC AGGTGGTCTG
3451 GGGACAGAGT TCTCCGAGCT GCCTGGGAAG AGCAGAGACC CTTGGgAGCC
3501 TCCCAGGGAG GGTAGGGAGG AGTCAgAGGC TGAGGCCCCC AGGGGAGCAG
3551 AGGAGGCGTT CCCTGCTGAG ACCCTGGGCC ACACTGGAAG TGATGCCCCT
3601 TCACCTTGGC CTCTGGGGTC AGAGGAAGCT GAGGAGGATG TACCACCAGT
3651 GCTGGTCTCC CCCAGCCCAA CGTACACCCC GATCCTGGAA GATGCCCCTG
3701 GGCTrCAGCC TCAGGCTGAA GGGAGTCAGG AGGCTAGCTG GGGGGTGCAG
3751 GGGAGGGCTG AAGCTGGGAA AGTAGAGAGC GAGCAGGAGG AGTTGGGTTC
3801 TGGGGAGATC CCCGAGGGCC TCCAGGAGGA AGGGGAGGAG AGCAGAGAAG
3851 AGAGCGAGGA GGATGAGCTC GGGGAGACCC TTCCAGACTC CACTCCCCTG
3901 GGCTTCTACC TCAGGTCCCC CACCTCCCCC AGGTGGACCC CACTGGAGAG
3951 CAGAGGCCAC CCCCTCAAGG AGACTGGAAA GGAGGGCTGG GATCCTGCTG
4001 TCCTGGCTTC CGAGGGCCTT GAGGArCCCT CAGAAAAGGA GGAGGGGGAG
4051 GAGGGAGAAG AGGAGTGTGG CCGTGACTCT GACCTGTCAG AAGAATTTGA
4101 GGACCTGGGG ACTGAGGCAC CTTTTCTTCC TGGGGTCCCT GGGGAGGTGG

FIGURE 2 (Continued)

4151 CAGAACCTCT GGGCCAGgtg cCcCaGCTGC TACTGGATCC TGCAGCCTGG
4201 GATCGAGATG GGGAGTCTGA TGGGTTTGCA GATGAGGAAg AAAGTGGGGA
4251 GGAGGGAGAG GAGGATCAGG AGGAGGGGAG GGAGCCAGGG GCTGGGCGGT
4301 GGGGGCCAGG GTCTTCTGTT GGCAGCCTCC AGGCCCTGAG TAGCTCCCAG
4351 AGAGGGGAAT TCCTGGAGTC TGATTCTGTA AGTGTCAGCG TCCCCTGGGA
4401 TGACAGCTTG AGGGGTGCAG TGGCTGGTGC CCCCAAGACT GCCCTGGAAA
4451 CGGAGTCCCA GGACAGTGCT GAGCCTTCTG GCTCAGAGGA AGAGTCTGAC
4501 CCTGTTTCCT TGGAGAGGGA GGACAAAGTC CCTGGCCCTC TAGAGATCCC
4551 CAGTGGGATG GAGGATGCAG GCCCAGGGGC AGACATCATT GGTGTTAATG
4601 GCCAGGGTCC CAACTTGGAG GGGAAGTCAC AGCATGTAAA TGGGGGAGTA
4651 ATGAACGGGC TGGAGCAGTC TGAGGAAAGT GGGGCAAGGA ATGCGCTAGT
4701 CTCTGAGGGA GACCGAGGGA GCCCCTTTCA GGAGGAGGAG GGGAGTGCTC
4751 TGAAGAGGTC TTCGGCAGGG GCTCCTGTTC ACCTGGGCCA GGGTCAGTTC
4801 CTGAAGTTCA CTCAGAGGGA AGGAGATAGA GAGTCCTGGT CCTCAGGGGA
4851 GGAC

FIGURE 3

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   1 MEGCMGEESF QMWELNRRLE AYLGRVKALE EQNELLSAGL GGLRRQSADT
  51 SWRAHADDEL AALRALVDQR WREKHAAEVA RDNLAEELEG VAGRCEQLRL
 101 ARERTTEEVA RNRRAVEAEK CARAWLSSQG AELERELEAL RVAHEEERVG
 151 LNAQAACAPR LPAPPRPPAP APEVEELARR LGEAWRGAVR GYQERVAHME
 201 TSLDQTRERL ARAVQGAREV RLELQQLQAE RGGLLERRAA LEQRLEGRWQ
 251 ERLRATEKFQ LAVEALEQEK QGLQSQIAQV LEGRQQLAHL KMSLSLEVAT
 301 YRTLLEAENS RLQTPGGGSK TSLSFQDPKL ELQFPRTPEG RRLGSLLPVL
 351 SPTSLPSPLP ATLETPVPAF LKNQEFLQAR TPTLASTPIP PTPQAPSPAV
 401 DAEIRAQDAP LSLLQTQGGR KQAPEPLRAE ARVAIPASVL PGPEEPGGQR
 451 QEASTGQSPE DHASLAPPLS PDHSSLEAKD GESGGSRVFS ICRGEGEGQI
 501 WGLVEKETAI EGKVVSSLQQ EIWEEEDLNR KEIQDSQVPL EKETLKSLGE
 551 EIQESLKTLE NQSHETLERE NQECPRSLEE DLETLKSLEK ENKRAIKGCG
 601 GSETSRKRGC RQLKPTGKED TQTLQSLQKE NQELMKSLEG NLETFLFPGT
 651 ENQELVSSLQ ENLESLTALE KENQEPLRSP EVGDEEALRP LTKENQEPLR
 701 SLEDENKEAF RSLEKENQEP LKTLEEEDQS IVRPLETENH KSLRSLEEQD
 751 QETLRTLEKE TQQRRRSLGE QDQMTLRPPE KVDLEPLKSL DQEIARPLEN
 801 ENQEFLKSLK EESVEAVKSL ETEILESLKS AGQENLETLK SPETQAPLWT
 851 PEEINKSGGN ESSRKGNSRT TGVCGSEPRD IQTPGRGESG IIEISGSMEP
 901 GEFEISRGVD KESQRNLEEE ENLGKGEYQE SLRSLEEEGQ ELPQSADVQR
 951 WEDTVEKDQE LAQESPPGMA GVENKDEAEL NLREQDGFTG KEEVVEQGEL
1001 NATEEVWFPG EGHPENPEPK EQRGLVEGAS VKGGAEGLQD PEGQSQQVGT
1051 PGLQAPQGLP EAIEPLVEDD VAPGGDQASP EVMLGSEPAM GESAAGAEPG
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FIGURE 3 (Continued)

1101 LGQGVGGLGD PGHLTREEVM EPPLEEESLE AKRVQGLEGP RKDLEEAGGL

1151 GTEFSELPGK SRDPWEPPRE GREESEAEAP RGAEEAFPAE TLGHTGSDAP

1201 SPWPLGSEEA EEDVPPVLVS PSPTYTPILE DAPGLQPQAE GSQEASWGVQ

1251 GRAEAGKVES EQEELGSGEI PEGLQEEGEE SREESEEDEL GETLPDSTPL

1301 GFYLRSPTSP RWTPLESRGH PLKETGKEGW DPAVLASEGL EEPSEKEEGE

1351 EGEEECGRDS DLSEEFEDLG TEAPFLPGVP GEVAEPLGQV PQLLLDPAAW

1401 DRDGESDGFA DEEESGEEGE EDQEEGREPG AGRWGPGSSV GSLQALSSSQ

1451 RGEFLESDSV SVSVPWDDSL RGAVAGAPKT ALETESQDSA EPSGSEEESD

1501 PVSLEREDKV PGPLEIPSGM EDAGPGADII GVNGQGPNLE GKSQHVNGGV

1551 MNGLEQSEES GARNALVSEG DRGSPFQEEE GSALKRSSAG APVHLGQGQF-

1601 LKFTQREGDR ESWSSGED